Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Testing for Preservative Interference with Sterility Tests

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Contact Person: Gerald G. Christianson, (515) 663-7416

Approvals:

_____ Date:_____
Gerald G. Christianson, Head
Sterility/Extraneous Agents Section

_____ Date:_____
Ann L. Wiegers, Quality Assurance Manager

_/s/ Randall L. Levings_____ Date:_11/05/99
Randall L. Levings, Director
Center for Veterinary Biologics-Laboratory

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

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Supplemental Assay Method for Testing for Preservative Interference with Sterility Tests

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) tests for preservative interference with sterility tests and determines the ratio of inoculum to medium which results in sufficient dilution to prevent bacteriostatic and fungistatic activity. This testing is required by the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(d).

1.2 Keywords

Preservative interference, vaccines, bacterins, bacteriostatic, fungistatic

2. Materials

2.1 Equipment/instrumentation

2.1.1 30°-35°C incubator

2.1.2 20°-25°C incubator

2.1.3 Sterile disposable cotton-plugged pipettes

2.1.4 Sterile 10-ml disposable syringes with needles

2.1.5 Biosafety cabinet

2.1.6 Vortex mixer

2.1.7 Water bath

2.2 Reagents/supplies

2.2.1 Indicator Organisms: Use *Bacillus subtilis* (American Type Culture Collection [ATCC] #6633), *Candida krusei (*ATCC #6258), and *Clostridium chauvoei* spores, or equivalent organisms as specified in the current United States Pharmacopoeia (USP), as the

indicator organisms to determine the preservative interference according to 9 CFR, Part 113.25(d).

2.2.2 Media: Brain Heart Infusion Agar (BHIA), Soybean-Casein Digest Agar (SCDA) or Trypticase Soy Agar (TSA), Soybean-Casein Digest Medium (SCDM) or Trypticase Soy Broth (TSB), Fluid Thioglycollate Medium (FTM), and Fluid Thioglycollate Medium with 0.5% Beef Extract (FTM/BE). See **Section 9.1** for Media Formulations.

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel performing the test must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn biosafety cabinets on at least 1 hr before preparing positive control reagents or testing media for growth promotion.

3.2.2 Monitor incubators daily for temperature according to the current version of GDOCSOP0001.

3.2.3 Monitor freezers and coolers used for the storage of reagents and controls for temperature daily according to the current version of GDOCSOP0003.

3.3 Preparation of reagents

3.3.1 Use frozen (-70°C) or lyophilized stock cultures of *B. subtilis* spores and *C. krusei,* prepared according to the current version of STSAM0900 **(Sections 4.1** and **4.2)**, as a source of indicator organisms. Use a frozen (-70°C) stock culture of *C. chauvoei* spores, prepared as described in the current version of STSAM0901 (**Section 3.3**) as the source of this indicator organism. Prepare suspensions of the indicator organisms and then determine the number of indicator organisms per ml of suspension by plate count as described in the current version of STSAM0902 **(Section 4.1)** for *B. subtilis* and *C. krusei*. Determine the number of organisms per ml for *C. chauvoei* by most probable numbers (MPN). The procedure is described as the most probable total count by multiple-tube method in the 1985 U.S. Pharmacopeia XXI, page 154.

3.3.2 To test for preservative interference from aerobic bacterins and killed virus biologics, use *B. subtilis* with FTM at 30°-35°C and *C. krusei* with SCDM and FTM at 20°-25°C. For anaerobic bacterins, use *C. chauvoei* with FTM/BE at 30°-35°C and *C. krusei* with SCDM and FTM at 20°-25°C. For parenteral live viral products, use *B. subtilis* with SCDM at 30°-35°C and *C. krusei* with SCDM at 20°-25°C. For chicken embryo origin (CEO), poultry, drinking water vaccines, use *B. subtilis* with BHIA at 30°-35°C and *C. krusei* with BHIA at 20°-25°C.

4. Performance of the test

4.1 Broth media

4.1.1 Prepare 20 test vessels containing the specified test media for the type of biologic product being tested **(Section 3.3.2**) for each indicator organism.

Row 1 (10 test vessels)
Row 2 (10 test vessels)

4.1.2 Row 1 is the control. Inoculate each test vessel in row 1 with approximately 100 CFU of the indicator organism.

4.1.3 Row 2 is the test. Inoculate each test vessel in row 2 with 1 ml or 0.2 ml of the test sample, depending on the sterility test (9 CFR 113.26 or 113.27). Next, inoculate each test vessel in row 2 with approximately 100 CFU of the indicator organism.

4.1.4 Incubate the test vessels at the appropriate temperature, depending on the indicator organism used, for 14 days and observe for growth.

4.2 Agar media

4.2.1 When poured agar plates are used to test CEO drinking water vaccine, label 20 empty petri dishes for each indicator organism.

4.2.2 Inoculate each of 10 petri dishes with 10 bird doses of product. All 20 petri dishes are then inoculated with approximately 100 CFU of the indicator organism.

4.2.3 Pour melted BHIA, which has been cooled to 60^{0}C or less, onto each of the 20 petri dishes. The petri dishes are then swirled to mix the product and the 100 CFU of the indicator organism.

4.2.4 The pour plates are incubated for 7 days at 30°-35°C if inoculated with *B. subtilis* and for 14 days at 20°-25°C if inoculated with *C. krusei.*

5. Interpretation of the test results

5.1 There is no interference in the broth test if the test vessels containing biologic have the same number of test vessels showing growth as their corresponding test vessels containing no biologic.

5.2 There is no interference in the pour plate agar test if the plates containing biologic have the same average CFU (± 20%) as their corresponding plates containing no biologic.

5.3 If interference is demonstrated by a reduced growth in the test vessels or plates containing biologic, the test procedure must be repeated using a larger volume of medium. A range of larger media volumes must be tried, after interference is noted, to eliminate multiple repeats of this testing.

6. Report of test results

Record the results of these preservative interference tests in the log book of the appropriate sterility or purity test code. The results are recorded on the facing page to the test record. Also record the results of these preservative interference tests in the Vollist data base on the sterility central computer.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.25(d), U.S. Government Printing Office, Washington, DC, 1999.

7.2 The U.S. Pharmacopeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, PA.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVBL QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. The following is a list of notable changes made from the superseded April 15, 1992, and March 1, 1990, versions.

8.1 The word “tube” was replaced by “test vessel” to allow the use of tubes, flasks or bottles for larger volumes of broth.

8.2 Equivalent organisms; *Clostridium sporogenes* (ATCC #11437), and *Candida albicans* (ATCC #10231) as specified in the USP can be substituted.

9. Appendices

9.1 Media formulations

9.1.1 NVSL Media Formulation No. 10204

BRAIN HEART INFUSION AGAR (BHIA)

Brain Heart Infusion Agar	52.0 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

9.1.2 NVSL Media Formulation No. 10487

TRYPTICASE SOY AGAR (TSA)
OR
SOYBEAN-CASEIN DIGEST AGAR (SCDA)

Trypticase Soy Agar	40.0 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

9.1.3 NVSL Media Formulation No. 10423

TRYPTICASE SOY BROTH (TSB)
OR
SOYBEAN-CASEIN DIGEST MEDIUM (SCDM)

Trypticase Soy Broth	30.0 g
QH_2O	1000 ml

Autoclave 20 min at 121°C.

TSB and SCDM are 2 names for the same media formulation from different media companies.

9.1.4 NVSL Media Formulation No. 10135

FLUID THIOGLYCOLLATE MEDIUM (BBL)

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 ml

Mix and heat to boiling. Autoclave 20 min at 121°C.

9.1.5 NVSL Media Formulation #10227

FLUID THIOGLYCOLLATE WITH BEEF EXTRACT

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 ml

Heat and add:

0.5% Beef Extract (Difco)	5.0 g

Bring to a boil and dispense. Autoclave 20 min at 121°C.